nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☒	☐	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☒	☐	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Leica application Suite X3.5.7.23225 for the imaging with Leica SP8 Zen3.1 (black version) for Light sheet microscopy Fusion2.3.0.44 for Dragonfly Quick PhotoMicro2.3 for Olympus SZX10 Cell Ranger Single-Cell Software Suite 6.1.1.
Data analysis	ZEN 3.4 (BLUE VESION) Imarisx64.9.6.0 Fiji Imagej1.54d Seurat package 4.3.0.1 R 4.2.1 Ubuntu 20.04.5 LTS

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The scRNAseq datasets generated in this study have been deposited in the ENA database under accession code "PRJEB67341" available at https://www.ebi.ac.uk/ena/browser/view/PRJEB67341.
The detailed code applied for the analysis in this study is available
here: https://codeocean.com/capsule/f000b9e2-6830-4935-9280-177e006ddf63/
and at https://github.com/jakubovciak/Vertebrate_Head

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	*Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design; whether sex and/or gender was determined based on self-reporting or assigned and methods used.* *Provide in the source data disaggregated sex and gender data, where this information has been collected, and if consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected.* *Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.*
Reporting on race, ethnicity, or other socially relevant groupings	*Please specify the socially constructed or socially relevant categorization variable(s) used in your manuscript and explain why they were used. Please note that such variables should not be used as proxies for other socially constructed/relevant variables (for example, race or ethnicity should not be used as a proxy for socioeconomic status).* *Provide clear definitions of the relevant terms used, how they were provided (by the participants/respondents, the researchers, or third parties), and the method(s) used to classify people into the different categories (e.g. self-report, census or administrative data, social media data, etc.)* *Please provide details about how you controlled for confounding variables in your analyses.*
Population characteristics	*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*
Recruitment	*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For the single cell analysis, the maximum possible number of embryos was chosen to ensure with high viability of single cells during trypsinization. For the analysis of expression patterns in wild-type embryos and experiments involving pharmacological treatment, we followed standard practices in the field. Each experiment involving pharmacological treatment was conducted at least three times, consistently yielding similar results. For gene expression analysis of pharmacologically treated amphioxus embryos, 10-20 treated embryos were used in the individual analysis, with a minimum of ten treated embryos analyzed using confocal microscopy and at least three imaged as a complete z-stack. For gene expression analysis of pharmacologically treated zebrafish embryos, 20-40 embryos were used in each experiment. Two independent germline founders were identified for each zebrafish transgenic line and in each case were verified to show similarly specific activity in at least 10 independent animals. In this study, sample sizes were primarily determined by empirical observations and the qualitative nature of our data. For gene expression analysis and related experiments, we typically used around 15 embryos per condition, a number demonstrated through extensive preliminary observations to reliably reveal consistent patterns across multiple

independent experiments.
We did not employ formal statistical methods to pre-determine sample sizes as our data are primarily pattern-based, not quantitative. The observed consistency of patterns across experiments serves as a practical indicator of reproducibility and reliability. For instance, a specific gene expression pattern observed in at least 13 out of 15 embryos, or 80% consistency, is considered statistically significant. This threshold aligns with standard practices in developmental biology, where visual and morphological consistencies provide substantial evidence of biological phenomena.

Data exclusions	No data were excluded from analysis
Replication	Each experiment involving pharmacological treatment was conducted at least three times. All attempts of replication were successful.
Randomization	The allocation of the samples was random.
Blinding	In our study blinding was not deemed relevant due to the nature of our research. We did not have expectations, our goal was to understand the inherent mechanisms of development as they occur in their natural context.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	The antibodies against Six3/6, Tbx1, IrxC, Islet were generated in mouse as previously described in: Bozzo, M., Pergner, J., Kozmik, Z. & Kozmikova, I. Novel polyclonal antibodies as a useful tool for expression studies in amphioxus embryos. Int J Dev Biol 61, 793-800, doi:10.1387/ijdb.170259ik (2017). The antibodies, which were used in previously published studies were: against Brachyury1, Elav and FoxA1(Kozmikova, I. & Kozmik, Z. Wnt/beta-catenin signaling is an evolutionarily conserved determinant of chordate dorsal organizer. Elife 9, doi:10.7554/eLife.56817 (2020)), Otx (1 Vopalensky, P. et al. Molecular analysis of the amphioxus frontal eye unravels the evolutionary origin of the retina and pigment cells of the vertebrate eye. Proc Natl Acad Sci U S A 109, 15383-15388, doi:10.1073/pnas.1207580109 (2012)). These primary antibodies were used at a dilution of 1:200. The commercial anti-Smad2 (phospho S255) antibody, produced in rabbit (Abcam, ab188334), was used at a dilution of 1:20,000. The secondary antibody, Alexa Fluor (Abcam), was used at a dilution of 1:500.
Validation	Antibodies were validated by confirming that their staining patterns were consistent with expression patterns observed through in-situ hybridization. An antibody was considered suitable for use when its staining replicated the expression pattern derived from in-situ hybridization. Specifically, for the Six3/6 antibody, the expression pattern from Kozmik, Z. et al. (https://doi.org/10.1016/j.ydbio.2007.03.009) was used as a reference. For Tbx1, we referred to Mahadevan, N. et al. (http://dx.doi.org/10.1007/s00427-004-0433-1), and for IrxC, Kaltenbach, C. et al. (http://dx.doi.org/10.1016/j.gep.2009.02.003) served as the comparison source.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	We used two different organisms in our study: 1)Branchiostoma floridae: Source: Laboratory culture Life Stage: Adults (used for spawning) and embryos (used for experiments) 2)Zebrafish (Danio rerio): Strain: Wild-Type (AB) Adults (used for obtaining embryos) and embryos (used for experiments)

Wild animals	The study did not involve wild animal
Reporting on sex	This information has not been collected. The experiments were performed on embryos.
Field-collected samples	The study did not involve the samples collected in the fields
Ethics oversight	Institute of Molecular Genetics of the Czech Academy of Sciences, Animal Welfare and Protection Committee. Membership: MVDr. Jan Honetschläger, MBA RNDr. Vít Karafiát Mgr. Jolana Turečková, Ph.D. Mgr. Jana Petrusová, Ph.D. Mgr. Lucie Janečková (Tůmová) RNDr. Martin Gregor, Ph.D.Mgr. Monika Šťastná, Ph.D. (Horázná) RNDr. Jarmila Vojtíšková, CSc. MVDr. Petra Králová Viziová, Ph.D. Mgr. Ondřej Svoboda, Ph.D. MVDr. Libor Kopkan, Ph.D. Ing. Martina Minariková Mgr. Jana Balounová, Ph.D. Mgr. Ondřej Svoboda, Ph.D. MVDr. Libor Kopkan, Ph.D. Ing. Martina Minariková Mgr. Jana Balounová, Ph.D.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	*Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.*
Novel plant genotypes	*Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.*
Authentication	*Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.*